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Characterisation and expression of phospholipases B from the opportunistic fungus *Aspergillus fumigatus*

Da-Kang Shen [a], Ali Dehghan Noodeh [b], Abdolhassan Kazemi [b], Renée Grillot [a,c], Geoff Robson [b], Jean-François Brugère [a,*]

[a] *Laboratoire Interactions Cellulaires Parasite-Hôte – ICPH, Facultéde Médecine et Pharmacie, Université Joseph Fourier, F-38706 La Tronche, France*

[b] *School of Biological Sciences, 1.800 Stopford Building, University of Manchester, Manchester M13 9T, UK*

[c] *Laboratoire de Parasitologie et Mycologie, CHU de Grenoble, 38700 La Tronche, France*

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Abstract

The phospholipase B family (PLB) are enzymes sharing phospholipase (PL), lysophospholipase (LPL) and lysophospholipase-transacylase (LPTA) activities. They have been shown to be important virulence factors in several human fungal pathogens including *Candida albicans* and *Cryptococcus neoformans*. *Aspergillus fumigatus*, a human opportunistic fungal pathogen leading to a high rate of mortality in immunosuppressed patients is known to possess an extracellular phospholipase B activity. In this paper, we report the molecular characterisation of three PLB genes from *A. fumigatus* (af*plb*) using degenerate primers in PCR amplification and data from the *A. fumigatus* genome project. They are expressed at 37 °C, and two of them (af*plb1* and af*plb3*) are induced by lecithin. They encode proteins of 633, 588 and 630 amino acids, respectively, presenting together a T-Coffee score of 81. They also possess the amino acid triad responsible for enzymatic activity in the mammalian cytosolic PLA_2 and other fungal PLBs. AfPLB1 and afPLB3 are secreted with a cleaved signal peptide. The complete cDNA sequences were obtained by RACE-PCR for the two secreted afPLBs and probably account for the extracellular phospholipase activity previously reported in the culture media of *A. fumigatus*.

Keywords: Aspergillosis; Lysophospholipase; Phospholipase B family; Pathogenic fungus; Virulence factor

1. Introduction

Aspergillus fumigatus is one of the most prevalent opportunistic human fungal pathogens, the number of cases having increased together with the number of immunocompromised individuals. The most serious form of *Aspergillus* infection, invasive aspergillosis, has been found in 4% of all patients dying in a modern European teaching hospital [1]. Pathogenesis factors are thought to include toxins, proteins facilitating adhesion, and hydrolases acting on host cell components (reviewed in [2,3]). These include proteases [4] and probably phospholipases. Phospholipases are a heterogeneous group of enzymes that are able to hydrolyse one or more ester linkages in glycerophospholipids and include phospholipase A (PLA), B (PLB), C (PLC) and D (PLD). Phospholipase activity can destabilize

* Corresponding author. Tel.: +33 4 76 63 74 73; fax: +33 4 76 63 74 73.

E-mail address: Jean-Francois.Brugere@ujf-grenoble.fr (J.-F. Brugère).

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host membranes, lyse cells and release lipid second messengers [5]. They are considered to be important virulence factors for many microorganisms including *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Rickettsia rickettsii*, *Toxoplasma gondii* and *Entamoeba histolytica* (review in [6]). Phospholipases and particularly PLBs are also considered to be virulence factors for pathogenic fungi including *Candida albicans* [7] and *Cryptococcus neoformans* [8]. In fungi, these PLBs possess three different enzymatic activities, the hydrolase activity of phospholipase, lysophospholipase (LPL) and a lysophospholipase transacylase (LPTA) activity [7,9]. Extracellular phospholipase activities have been detected in in-vitro cultures of *A. fumigatus* [10,11], with a predominance of PLB activities [12] supporting the presence of gene(s) coding for secreted PLB(s). We therefore decided to identify gene(s) and cDNA(s) responsible of this activity in order to determine their potential role in the pathogenesis of *A. fumigatus*.

2. Materials and methods

2.1. Strains, media and culture conditions

A. fumigatus CBS14489 and AF10 (ATCC 90240) were maintained at 4 °C on Sabouraud chloramphenicol agar (bioMérieux, France) and subcultured at 25 or 37 °C up to 48 h with constant shaking (200 rpm) in modified Czapeck broth (Cz_2) with or without 0.5% (w/v) 3-Sn-phosphatidylcholine from egg yolk (Fluka, Switzerland). Cz_2 consists of 0.1% (w/v) yeast extract, 0.3% (w/v) saccharose, 0.3% (w/v) potassium nitrate, 0.1% (w/v) di-potassium hydrogen phosphate, 0.05% (w/v) potassium chloride, 0.05% (w/v) magnesium sulphate and 0.001% (w/v) iron (II) sulphate.

2.2. DNA and RNA extraction

Genomic DNA was extracted as described on the *Aspergillus* website (www.aspergillus.man.ac.uk). Total RNA was extracted using a TRI REAGENT™ Kit (Euromedex, France) according to the manufacturer's instructions. Isolation of mRNA was accomplished by using "Dynabeads® mRNA DIRECT™ Kit" (Dynal, Norway) on total RNA according to the manufacturer's instructions.

2.3. Cloning and characterisation of af*plb genes*

Degenerate primers for phospholipase B [13] named degPLB Fw and Rv (Table 1) were used in PCR amplification. The final optimised conditions were 4 mM $MgCl_2$ and an annealing temperature of 48 °C for 3 min. Cloning of PCR products were performed either in pGEM®-T easy (Promega) or pT-Adv (Invitrogen) according to the manufacturer's instructions. *Xho*I-restricted genomic DNA or *Sal*I-restricted DNA was self-ligated with T4 DNA ligase and used for determination of adjacent sequences by Inverse PCR [14], respectively, for af*plb1* and af*plb2*. The sequence of the primers used (invPLB1 and 2, Fw and Rv) is given in Table 1.

Table 1
Names and sequences of primers used in this work

Name	Sequence (5′–3′)
degPLB Fw	GAYGGIGGIGARAAYCARAA [13]
degPLB Rv	AYIGTICCRTTCCARCARTA [13]
invPLB1 Fw	CGCACACAGCTCTCATTACTTGGG
invPLB1 Rv	GGGAATACATCACGTGCTTATGAGGC
invPLB2 Fw	CTGGTAGTCGTGTCCGCTGACGAGTCGAC
invPLB2 Rv	TGAGAATGGGTACGATGTGGCAACACTGGG
RT-PLB1 Fw	ATACCACTGCACCCGTTGA
RT-PLB1 Rv	GGGAATTGCAGGAAAGGAA
RT-PLB2 Fw	CTCGATCCCCTTCTTCAGC
RT-PLB2 Rv	GCCAGTCGCGTTTGAACTA
RT-PLB3 Fw	TGCCAGACGTGAACACCTT
RT-PLB3 Rv	CCGCGTCACCACGTTATAC
RT-βACT Fw	TGATTGGTATGGGCCAGAA
RT-βACT Rv	CGTAGAGGGAGAGAACGGC
Q-PCR PLB1 Fw	TGTTGGCTTTGTCATGGGTA
Q-PCR PLB1 Rv	GATGTCGGTGAAGACGGATT
Q-PCR PLB2 Fw	CAGGCATTTCTGCAGATGAA
Q-PCR PLB2 Rv	GGCTCGCATAGCGATAGAAC
Q-PCR PLB3 Fw	CCAATGGAGCTGGAGCTATC
Q-PCR PLB3 Rv	TGCAGTAGACCACCGAGATG
Q-PCR ACT Fw	TGCTCCTCCTGAGCGTAAAT
Q-PCR ACT Rv	ACATCTGCTGGAAGGTGGAC
Q-PCR β-TUB Fw	ACTTCCGCAATGGACGTTAC
Q-PCR β-TUB Rv	GGATGTTGTTGGGAATCCAC
5′-RACE PLB1	GTTCCTAGATACTCGAGGGGCGCA
3′-RACE PLB1	TGCGCCCCTCGAGTATCTAGGAAC
5′-RACE PLB3	GGTGGAGTTGCGGTACCCGTAGAA
3′-RACE PLB3	TTCTACGGGTACCGCAACTCCACC

2.4. Expression of af*plbs and full-length cDNA cloning and sequencing*

Potential expression of PLB(s) was evaluated by RT-PCR on total RNAs (ProSTAR™ First-strand RT-PCR, Stratagene) using an annealing temperature of 58 °C. Primers used in PCR (RT-PLBx primers) are shown in Table 1. Amplification of a part of the cDNA of the housekeeping genes actin and β-tubulin was used as a positive control and to normalise the expression levels of the PLB genes between the cultures with and without lecithin. Quantitative-PCR (Q-PCR) was performed on a BioRad i-Cycler IQ system using the IQ SYBR Green supermix Kit (BioRad) according to the manufacturer's instructions, relatively to β-actin mRNAs. Primers used are indicated in Table 1.

5′- and 3′ regions of each cDNA were amplified by RACE-PCR (Rapid Amplification of cDNA Ends) accordingly to the manufacturer's instructions (Gene-

Racer™ Kit Invitrogen) using a set of gene specific primers (5′- or 3′-RACE PLBx, Table 1), with two rounds of PCR, one of 30 cycles with an annealing temperature of 65 °C and another 30-cycles at 68 °C. For each gene, the 5′ and the 3′ primers are complementary, therefore permitting full-length cDNA sequences to be assembled after sequencing (GENOME express, Meylan, France).

2.5. Sequence analysis and sequence Accession Numbers

Preliminary genomic sequence data was obtained from The Institute for Genomic Research (TIGR) website at www.tigr.org. Bioinformatics analyses were performed either at the French bioinformatics server "Infobiogen" (www.infobiogen.fr) or at the ExPASy Molecular Biology Server (http:us.expasy.org). For alignments and phylogenic analysis, the following PLBs sequences were used: YMR008C (scPLB1), YMR006C (scPLB2), YOL011W (scPLB3), AAF65220 (cnPLB1), CAC86376 (cnPLBa), AAF61964 (cnPLBb), EAL17514 (cnPLBx), Q9UWF6 (caPLB1), O93795 (caPLB2), Q9UVX1 (caPLB3), Q8TG07 (cgPLB1), Q8TG06 (cgPLB2), P78854 (spPLB1), O13857 (spPLB2), Q08108 (spPLB3), Q9P327 (spPLB4), Q9Y7N6 (spPLB5), Q9UTH5 (spPLB6), EAA64795 (anPLBx1), EAA61580 (anPLBx2), O42790 (ncPLB), EAA34954 (ncPLBx), EAA56932 (mgPLBx), EAK81777 (umPLBx).

The nucleotide sequence data reported in this paper are listed in the GenBank nucleotide sequence database under Accession Nos. AF223004/AF223005, respectively, for partial genomic sequences of af*plb1* and af*plb2*, and AY376592/AY376593, respectively, for the complete cDNA sequences of af*plb1* and af*plb3*.

3. Results

3.1. Determination of three plb genes in the A. fumigatus genome

Degenerate primers designed by Sugiyama et al. [13] for the cloning of a second phospholipase B gene from *C. albicans* were used in order to amplify a partial region of *plb* gene(s) from genomic DNA of *A. fumigatus*. A band of ~550 bp was obtained, corresponding after sequencing to two different sequences of 545 and 542 nt, sharing 65.9% identity and having a strong homology to fungal PLBs. These two partial sequences were therefore named af*plb1* (Genbank Accession No. AF223004) and af*plb2* (Genbank Accession No. AF223005). An inverse-PCR [14] on genomic DNA digested, respectively, by *Xho*I and *Sal*I and self-ligated permitted to obtain new PCR products of ~1.8 and ~1.1 kb leading to sequences of 2197 bp and of 1529 bp, respectively, for af*plb1* and af*plb2* when assembled (data not shown). However, a BLASTX analysis [15] revealed that while the entire 3′ ends of af*plb1* and af*plb2* were cloned, the 5′ regions were missing.

At the same time, the "*A. fumigatus* genome project" was initiated and preliminary data were available for the scientific community (http://www.tigr.org) from The Institute for Genomic Research (TIGR, USA) and the Wellcome Trust Sanger Institute (UK), the two main centres in this international collaboration [16]. We therefore decided to perform a BLAST search of our sequences to attempt to obtain the 5′ regions. For each sequence, a nearly perfect match was obtained confirming these genes were present as a single copy, which was also confirmed by southern analysis (data not shown). In addition, a third gene with high homology was identified indicating that the same number of *plb* genes was present in *A. fumigatus* genome and in *S. cerevisiae* genome.

3.2. Expression and cDNA sequences of afplbs

The main site of infection of *A. fumigatus* before invasion is the lung, in which the surfactant is principally composed of dipalmitoyl phosphatidylcholine, also the preferred substrate of the cryptococcal PLB [17]. We therefore investigated the influence of lecithin on the expression of af*plb* genes by quantifying their mRNAs from *A. fumigatus* cultured at 37 °C with or without lecithin. Real-time PCR indicates that the three af*plbs* are expressed and that af*plb1* and af*plb3* are up-regulated by lecithin (respectively, 5-fold and 300-fold) whereas af*plb2* expression is not affected (Table 2).

A preliminary bioinformatics study on the predicted amino acid sequence of PLB1, PLB2 and PLB3 showed the presence of an N-terminal prepro sequence for PLB1 and PLB3 (but not PLB2) indicating that they were likely to be secreted and therefore potentially involved in the pathogenesis of *A. fumigatus*. We therefore cloned the full-length cDNAs of af*plb1* and af*plb3* by

Table 2
Influence of lecithin on gene expression of afPLBs[a]

Gene	Expression level[a]		Fold induction
	−Phospholipid	+Phospholipid	
af*plb1*	0.3 ± 0.1	1.4 ± 0.2	5
af*plb2*	1.0 ± 0.2	1.0 ± 0.2	0
af*plb3*	<0.0001	0.04 ± 0.01	>300

[a] The expression of af*plbs* mRNA was determined by real-time PCR after extraction of mRNAs from *A. fumigatus* cultured at 37 °C for 48 h in Cz2 medium supplemented with (+) or without (−) 0.2% (w/v) lecithin. Results (means of six replicates) were normalized to β-actin and are given relative to its expression. The experiment was performed twice with similar results.

RACE-PCR using mRNA from *A. fumigatus* cultured at 37 °C in the presence of lecithin. Assembling of the 5′ and 3′ part of each sequence revealed an mRNA of 2125 nt for af*plb1* and of 2027 nt for af*plb3*, excluding the poly(A) tail, deposited in Genbank, respectively, under the Accession Nos. AY376592 and AY376593. These sequences were aligned and compared with genomic data. The ORF for af*plb2* was deduced from genomic data using bioinformatics tools (GeneFinder, TblastX) and sequence alignment against other fungal *plbs*. The features of these mRNAs are summarized in Table 3. Af*plb1* and af*plb2* are composed of 3 exons, against one for af*plb3* and the introns are relatively small (varying from 58 to 132 nt) and fit with the consensus sequence usually observed (GT/YAG). Search of the putative translation initiation codon (AUG) beginning the longest ORF sharing homology with other fungal PLBs for af*plb1* and af*plb3* reveals that the 5′ UTR is 63- and 34-nt long, respectively, for af*plb1* and af*plb3*. The stop codon used is OCH for af*plb1* and AMB for af*plb2* and af*plb3*. The 3′ UTR is 160- and 101-nt long, respectively, for af*plb1* and af*plb3*, so resulting of an ORF size of, respectively, 1899, 1764 and 1890 nt for af*plb1*, af*plb2* and af*plb3*.

3.3. Analysis of the predicted proteins sequences

The ORFs determined above lead to proteins of 633 amino acids for afPLB1, 588 amino acids for afPLB2 and of 630 amino acids for afPLB3. Their theoretic mass and pI are indicated in Table 3. An hydropathy analysis performed according to Kyte and Doolittle [18] revealed two stretches of hydrophobic amino acids at both the N- and C-terminus having a score superior of 2 (Table 3) only for afPLB1 and afPLB3, but not for afPLB2 which is predicted to be localized in the nucleus as indicated by a PSORT II analysis (60.9%, $k = 23$, [19]). In addition to the hydrophobic N-tails of afPLB1 and afPLB3, a secretion signal peptide is detected with a cleavage site between position 20 and 21 (VSG-AP) for afPLB1 and position 16 and 17 (ATA-TP) for afPLB3, using SignalP V2.0 with predictions trained on eukaryotes using neural networks [20]. Moreover, a PSORT II analysis reveals that these two proteins could be either extracellular (including the cell wall) or GPI anchored, with the same probability (Table 3). This last hypothesis is due to the presence of (1) a hydrophobic tail, (2) a N-terminal peptide for secretion, (3) a S/T rich region, and (4) a potential ω site (GPI attachment site) with a G [21], elements

Table 3
Main features of the sequences of the cDNA and of the deduced protein of afPLBs

		afPLB1	afPLB2	afPLB3
cDNA	cDNA size	4826	4951	4925
	Exons	3	3	1
	Size of exons	114 nt	ND	4925
		657 nt	566 nt	
		1354 nt	ND	
	Introns	2	2	0
	Size of introns	102 nt	132 nt	–
		58 nt	59 nt	
	(Genomic)- +1[a]	(t)-A	ND	(c)-A
	5′ UTR length	63 nt	ND	34 nt
	3′ UTR length	160 nt	ND	101 nt
	ORF size	1899 nt	1764 nt	1890 nt
	STOP codon	UAA	UAG	UAG
Protein	Predicted size[b]	633 aa	588 aa	630 aa
	Predicted mass[b]	68,143 Da	63,370 Da	67,448 Da
	Theoretical p*I*[b]	4.59	5.11	5.22
	Hydrophobicity[c]	N and C ter	None	N and C ter
	Secretion signal[d]	Yes	No	Yes
	Cleavage sequence[d]	..VSG_{20}/A_{21}P..	–	..ATA_{16}/T_{17}P..
	Resulting mass[b]	66,202 Da	–	65,893 Da
	Resulting p*I*[b]	4.55	–	5.14
	Localization[e]	EC/PM	N	EC/PM
	Probability[e] (%)	34.8/34.8	60.9	34.8/34.8
	N-glycosylation[f]	18	–	14

[a] The genomic nucleotide preceding the transcription initiation site (+1) is indicated in bracket when determined.
[b] Physical properties of deduced proteins (size, mass, p*I*) were determined using ProtParam at the ExPASy web Site.
[c] Hydropathy analysis was performed accordingly to Kyte and Doolittle [18].
[d] Secretion signal and cleavage sequence were determined using SignalP v2.0 [20].
[e] Localization is deduced from a PSORT II analysis [19]. EC stands for a extracellular position (including a cell wall localization), N for a nuclear position and PM for a plasma membrane localisation.
[f] *N*-glycosylation sites (N-{p}-[ST]) were determined using NetNGlyc 1.0 program.

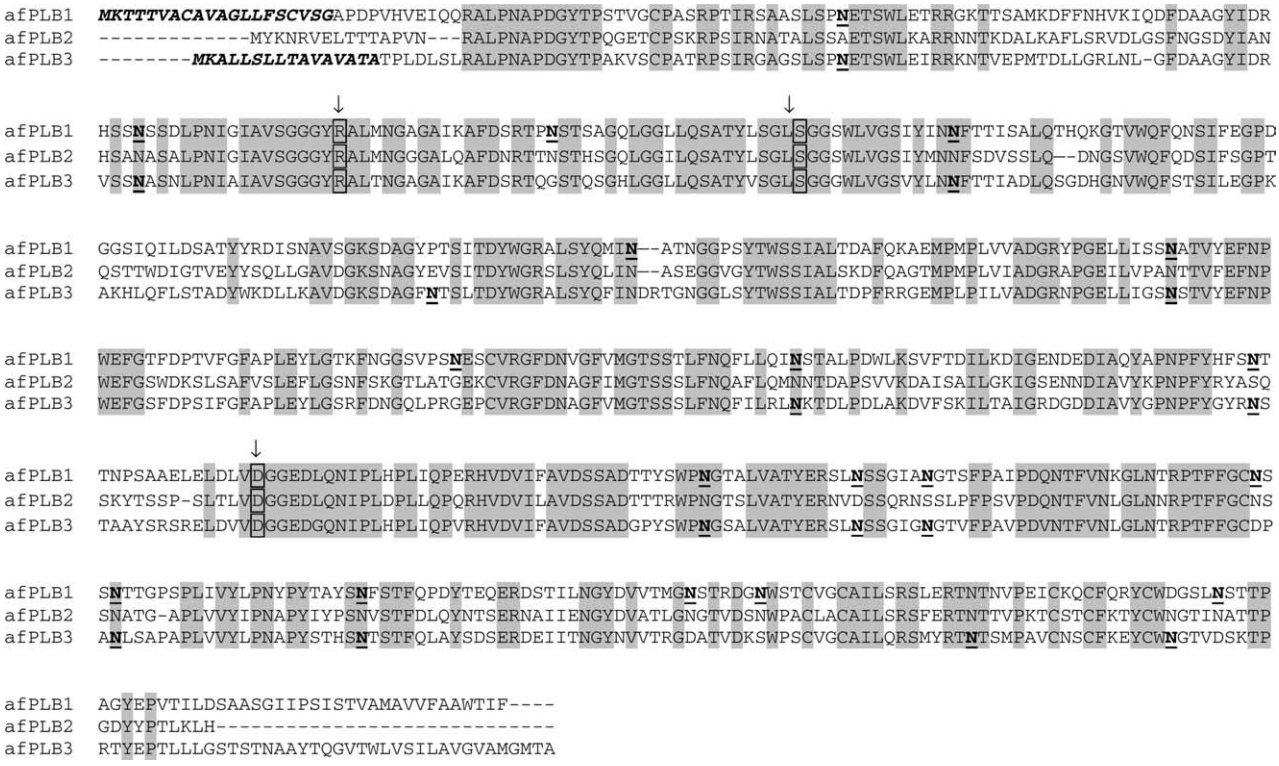

Fig. 1. Alignment of the three afPLBs. The secretion signal peptide of afPLB1 and afPLB3 is indicated in bold and italized. *N*-glycosylation sites (N-{p}-[ST]) are indicated in bold. Amino-acids of the catalytic triad identified in mammalian cytosolic PLA_2 are boxed and indicated by an arrow above them.

absent in caPLBs. These signal peptides are indicated in the Fig. 1, together with the important number of *N*-glycosylation sites present in these two secreted proteins (18 for afPLB1 and 14 for afPLB3).

An alignment of the sequence of these three proteins indicates a high degree of identities (from 59% to 63%), confirmed with a score of 81 using T-Coffee software [22]. Moreover, some residues of the cytosolic mammalian PLA_2 (a triad comprising R_{200}, S_{228} and D_{549} described as essential for the catalytic activity [23]) are also conserved in these three afPLBs (amino acids boxed in Fig. 1), at position 121/105/112 for R, 160/144/151 for S and 412/395/405 for D, respectively for afPLB1, afPLB2 and afPLB3. This is also the case for other PLBs from pathogenic fungi *C. albicans* (caPLB1 and caPLB2) and of *C. neoformans* (cnPLB1) (data not shown).

In order to identify orthologues in other fungi, a phylogenic analysis was performed using PLBs from yeasts and filamentous fungi. The tree is presented in Fig. 2 and shows that afPLBs are more closely related together than with PLBs from other genus: orthologous genes are only found in the *Aspergillus nidulans* genome (af*plb1* and an*plb1*, af*plb3* and an*plb2*), but not at the present time in other fungal genomes. A duplication of a common ancestor gene gave two genes, one which evolved into afPLB2, the other which duplicated into two other genes (afPLB1 and afPLB3): these events seems to be quite recent as they are restricted to the *Aspergilli*.

4. Discussion

In this present study, we identified and cloned three genes coding for PLB from *A. fumigatus*, as it is in the genome of *S. cerevisiae*. However, the genomic distribution seems to be different between these two organisms. In *S. cerevisiae*, two of the three *plb* genes (sc*plb1* and sc*plb2*) are linked on the chromosome XIII around the position 280,000. This is not the case for af*plb* genes as deduced from the partial assembly of genomic sequence data. Moreover, the phylogenic analysis indicates that the af*plb*s genes are probably the result of gene duplications that are independent of those observed in other fungi, with the exception of *A. nidulans*.

Our cDNA sequences for af*plb1* and af*plb3* fit well with those from the genome project, with some minor changes: for af*plb1*, a transition (33T → C), a transversion (1982 A → T) and a single-base deletion (2048ΔT) are observed, and are all localized in untranslated regions. Five single-base modifications are detected for af*plb3*, including four transitions (A → G in positions 391, 1658, 1974 and 1886) and one transversion (242C → G). This should lead to three amino-acids differences (E_{70} → Q; D_{542} → N;

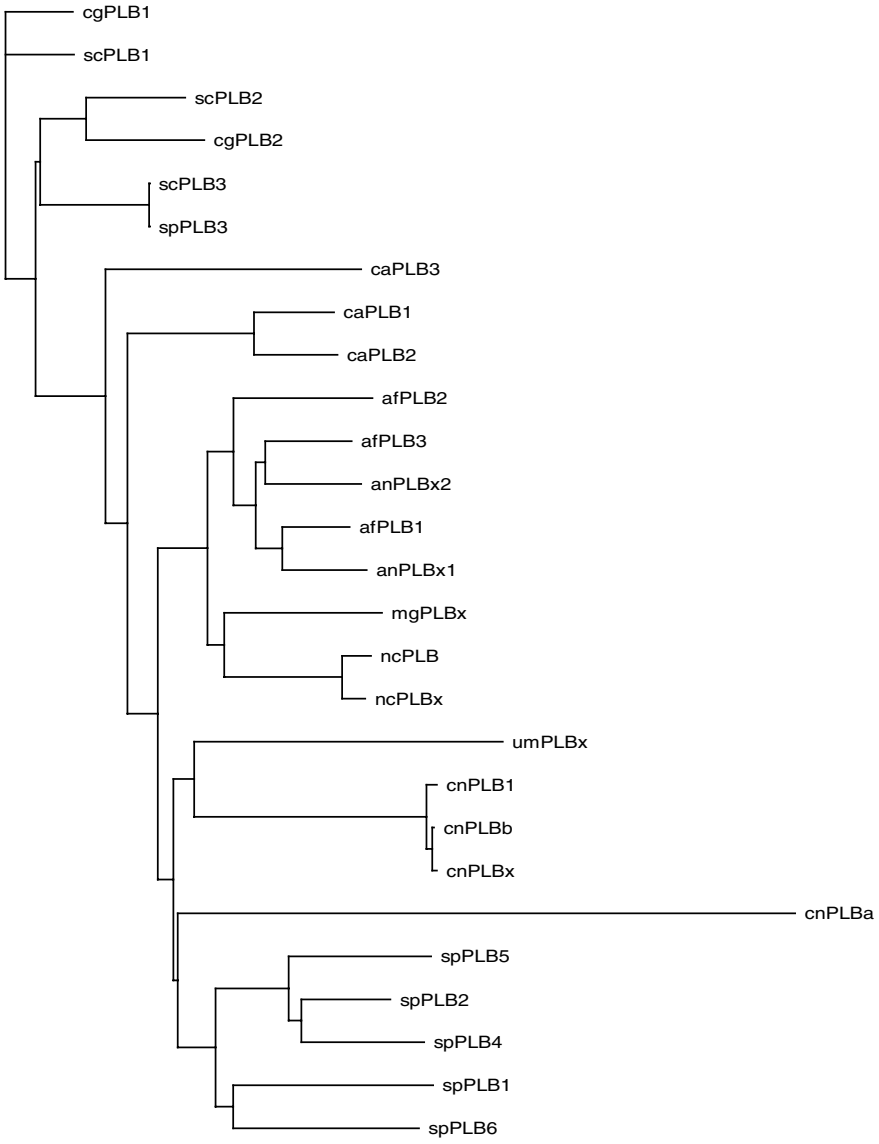

Fig. 2. Phylogenic tree of various fungal PLBs. The tree (unrooted tree) was drawn with TreeView, after a ClustalW alignment and a Phylip analysis using the Neighbor-joining method (x, hypothetical protein; af, *Aspergillus fumigatus*; an, *Aspergillus nidulans*, ca, *Candida albicans*; cg, *Candida glablatra*; cn, *Cryptococcus neoformans* ; mg, *Magnaporthe grisea*; nc, *Neurospora crassa*; um, *Ustilago maydis*). See Section 2 for Accession Numbers.

$S_{618} \rightarrow G$), the two other modifications being in an untranslated region (position 1974) or silent (position 391: G_{139}). It remains to be determined if these differences are sequencing errors, or more likely, genetic differences between the two different strains used in the genome project and in this study. Moreover, no canonical sequences of polyadenylation (AATAAA) were present in the 3′ part of the cDNAs and no consensus sequence for either a ''TATA box'' nor ''CAAT box'' could be identified in the 5′ upstream regions.

These genes encode PLBs which share 58–63% identity at the protein level and have similar properties to other fungal PLBs determined so far, as well for their size (varying from 605 aa for caPLB1 [7] to 637 aa for cnPLB1 [8]), and for their acidic pI or their high number of *N*-glycosylation sites. Interestingly, de-glycosylation of the PLB from *C. neoformans* using PNGase F leads to an almost total loss of enzyme activity [9]. They also possess the three amino acids responsible for their enzymatic activity. Two of them (afPLB1 and afPLB3) are non-cytoplasmic and possess an N-terminal signal peptide that should target the protein either to the plasma membrane or to the extracellular compartment (including an attachment to the cell wall). One or both proteins may therefore account for the extracellular PLB activity determined by Birch et al. [10,11] and would degrade the phospholipids present in high concentrations in lung surfactant. Interestingly, the expression of both afPLB1 and afPLB3 was induced by lecithin, a constituent of human lung surfactant. Extracellular PLB activity has previously been correlated with virulence in both *C. albicans* and *C. neoformans* [24,25] and PLB knock-

out strains have been shown to significantly reduce virulence by impeding penetration of host cells [7,8]. Moreover, it was shown recently that extracellular PLB activity is different in environmental isolates of *A. fumigatus* compared to clinical isolates [11], but surprisingly is higher. Thus, although PLB appears to be important in the pathogenicity of *C. albicans* and *C. neoformans*, it is less clear what role extracellular PLB may play in the pathogenicity of *A. fumigatus*. This may reflect the fact that while PLB appears to be the only secreted phospholipase in *C. albicans* and *C. neoformans*, *A. fumigatus* is also reported to secrete PLC [10,11]. However, we can not exclude a participation in virulence due to different substrate specificities.

In conclusion, we have demonstrated that *A. fumigatus* encodes three PLB genes that are predicted to encode two secreted PLB proteins and to be upregulated in the presence of lecithin. Gene knockout studies will be required to fully understand the potential role of PLB in the pathogenicity of *A. fumigatus*.

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